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TITLE: Research and Operational Support for the Study of Militarily Relevant Infectious Diseases of Interest in Both United States and Royal Thai Government

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Cooperative agreement # DAMD17-01-2-0005 was implemented January 1, 2001 to provide funding support for Royal Thai Army at Armed Forces Research Institute of medical Sciences (AFRIMS) engaged in research activities in collaboration with US Army. Administrative, logistical and scientific personnel required to support the ongoing US Army AFRIMS research efforts, and utilities and maintenance required to support the US Army AFRIMS research effort.

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FOREWORD

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S. Sangkharomaya February 5, 2003

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I. INTRODUCTION

A. General

Collaborative studies into infectious diseases of military importance have been conducted at the Armed Forces Research Institute of Medical Sciences (AFRIMS) by both the US Army Medical Component (USAMC) and the Royal Thai Army Medical Component (RTAMC) for 4 decades. Studies leading to develop drugs and vaccines to combat tropical diseases of military relevant importance.

B. Statement of work

Administrative, logistical and scientific personnel required to support the ongoing US Army AFRIMS research efforts, and utilities and maintenance required to support the US Army AFRIMS research effort.

C. US ARMY AFRIMS research efforts at Department of Entomology

Department of Entomology research efforts are the following:

1. Transmission-dynamics of anti-biotic resistant scrub typhus.

2. Field and laboratory evaluation of novel arthropod repellents against the vectors of malaria, dengue and scrub typhus in Thailand.

3. Use of a Geographic Information System (GIS) to implement and evaluate the efficacy of targeted vector control as a means of reducing malaria transmission.

4. Development of a chigger-challenge model for the evaluation of candidate scrub typhus vaccines.

5. Optimization of sporozoite production to support a human Plasmodium vivax sporozoite-challenge model.

D. US ARMY AFRIMS research efforts at Department of Immunology

Department of Immunology research efforts are the following:

See page 22-23

E. US ARMY AFRIMS research efforts at Department of Enteric Diseases

Department of Enteric Disearses research efforts are the following:

1. Surveillance of Diarrheal Diseases in Adult Travelers and Residents from Developed countries and Thai Adults: Etiology and Antibiotic Susceptibility Pattern Transmission-dynamics of anti-biotic resistant scrub typhus.

2. Surveillance of Diarrheal Diseases in Travelers and Resident Expatriates in Nepal: Etiology and Antibiotic Susceptibility Pattern.

3. Characterization of Enteric Pathogens isolated from Children in Hanoi.

4. Case-Control Study of Endemic Diarrhea in Children in Sangkhlaburi; along the Thai-Myanmar border.

5. Development and standardization of realtime PCR assays for detection and characterization of enteric pathogens.

6. Amplified Fragment Length Polymorphism (AFLP) fingerprint method to characterize enterotoxigenic E. coli (ETEC) colonization factors (CFs) and to study the genetic relationship among serotypes of Shigella flexneri.

7. Mechanisms of Antibiotic Resistance in Campylobacter jejuni

8. Stablishment of a non-human primate Campylobacter disease model prior to the pre-clinical evaluation of Campylobacter vaccine formulations.

9. Travelers' Diarrhea Among US Forces Deployed for Operation Cobra Gold

10. "Safety, dose, immunogenicity, and community transmission risk of a candidate s.flexneri 2a vaccine among young children in rural Bangladesh".

F. US ARMY AFRIMS research efforts at Department of Veterinary Medicine

Department of Veterinary Medicine research efforts are the following:

1. Efficacy and limited pharmacokinetic profiles of intravenous artemisinin candidates (artelinic acid (AL), artesunate (AS) or AL-like compounds) using a Plasmodium coatneyi - rhesus monkey model of severe malaria.

2. Toxicology of Single and Subacute Dosing of Candidate Intravenous Artemisinins in Rhesus Monkeys

3. Care and Maintenance of Rhesus (Macaca mulatta) and Cynomolgus (Macaca fascicularis) monkeys and Management of Breeding Colonies.

4. Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance / Quality Surveillance Program.

5. A Plasmodium berghei-Mouse Model for Screening Antimalarial Drugs.

G. US ARMY AFRIMS research efforts at Department of Virology

Department of Virology research efforts are following:

1. The Dengue Hemorrhagic Fever Project II: Continued Prospective Observational Studies of Children with Suspected Dengue

2. A Recombinant Hepatitis E Vaccine Efficacy Study In Nepalese Volunteers

3. Prospective Study of Dengue Virus Transmission and Disease in Primary School Children

4. Training and Workshops

5. Febrile Disease Surveillance, Kathmandu, Nepal

6. Hospital-based EID Surveillance, Kamphaeng Phet, Thailand

7. Influenza Surveillance in Southeast Asia

H. Space and Utilities Required

Funding under the cooperative agreement is also directed by the Principal Investigator to the provision of site maintenance including space and utilities management for both the RTAMC and the USAMC in support of research activities.

II. BODY

A. Department of Entomology, AFRIMS FY02 Research Accomplishments

1. Title of research project: Transmission-dynamics of anti-biotic resistant scrub typhus.

a. Investigators:

MAJ James W. Jones, PhD
Dr. Kriangkrai Lerdthusenee

b. Objectives:

Conduct an epidemiological investigation to determine the vectors of anti-biotic resistant scrub typhus. Scrub typhus is one of the most militarily relevant acarine-borne infectious diseases, with a long history of outbreaks in military populations in the CINPAC Area of Operations.

c. Methods:

This study was conducted in the vicinity of Chiangrai, in northern Thailand. Two field sites were selected: one closely associated with a foci of anti-biotic resistant scrub typhus and the other without reported anti-biotic resistance. In order to determine whether transmission of the anti-biotic resistant scrub typhus occurs only in the active rice field habitat, each field site was divided into 4 distinct habitats (village outskirts, active rice fields, ecotone, and wood/forest). In each habitat we established a grid system consisting of 100 traps (60 small live-traps, 30 medium live-traps, and 10 large live-traps). Collections were made on 4 consecutive days each month.

Blood and tissue samples and all ectoparasites (i.e., chiggers) were collected from all trapped animals. A sub-sample of each blood and tissue sample was assessed for the presence of O. tsutsugamushi by PCR, as were 25% of the chiggers collected from each animal. If a given sub-sample (blood, tissue or chigger) was positive, we subsequently attempted to isolate O. tsutsugamushi from the parent sample. In order to increase our success at isolating O. tsutsugamushi, we inoculated both laboratory mice and cell culture (L-929 cells) with triturated blood, tissue, or chiggers. Once O. tsutsugamushi was isolated from each PCR-positive sample, we assessed resistance to chloramphenicol, doxycycline, and azithromycin in laboratory mice. Groups of 10 mice each were inoculated with 100-LD50?s (calculated for each strain) or 5x106 infected cells of each O. tsutsugamushi isolate. By conducting experiments that inoculate both a known number of rickettsia (i.e. 5 million infected cells) as well as a known number of LD50?s we can control for variation in virulence/pathogenicity in the different isolates, while still quantifying anti-biotic resistance. Each group of mice were treated with serial dilutions of chloramphenicol, doxycycline, or azithromycin on days 5, 6, and 7, with control animals

receiving diluent only. The maximum dose given was approximately 6 times the human dose (300mg/kg/day, 18mg/kg/day, and 50mg/kg/day for chloramphenicol, doxycycline, and azithromycin, respectively) according to Watt et al. (1996). In addition to quantifying the degree of anti-biotic resistance for each isolate, we will also characterize strain variation in each isolate using the p56 and 16S rDNA portions of the genome. We will identify and characterize the field isolates using molecular diagnostic techniques (heteroduplex PCR and DNA sequencing). Results from the field isolates were compared with 8 "reference" strains (Karp, Kato, Gilliam, TH1817, TA 686, TA 678, TA 716, and TA 763).

d. Results (accomplishments during the period of January 2002 - December 2002):

From Feb. 01 to Nov. 2002, Approximately 2400 small mammals representing 19 species (6 orders) were collected. Predominant species were Bandicota indica , Rattus, rattus , and Rattus losea . Our FY02 total comprises over 1200 rodents. Approximately 38,000 chiggers were collected from these mammals, of which the primary species were Leptotrobidium imphalum and L. chiangraiensis. Of 2,031 rodents caught and tested, 108 were found positive for Orientia tsutusgamushi. Detection of O. tsutusgamushi. was most often recorded in the spleen, followed by liver kidney, and blood samples. Chigger specimens were removed from wild caught rodents and randomly selected for detection of the O. tsutsugamushi. Of 5000 chiggers removed from 230 rodents and tested for the presence of O. tsutsugamushi, 29 chiggers (from 23 rodents) were found positive. The majority were L. imphalum. We are in the process of completing characterization, using restriction fragment length polymorphism (RFLP) techniques, of O. tsutsugamushi isolates obtained from positive rodents. DNA sequencing of the entire 56-kD gene from these isolates is also in progress, about 800 out of 1500 base-pairs were successfully identified.

e. Future plans:

We plan to submit an NIH grant, "Antibiotic resistance in Scrub typhus" in collaboration with Dr. Adu Azad and Dr. Suzanna Radulovic at the University of Maryland.

2. Title of research project:Field and laboratory evaluation of novel arthropod repellents against the vectors of malaria, dengue and scrub typhus in Thailand.

a. Investigators:

MAJ James W. Jones, PhD; LTC Mustapha Debboun; Dr. Kriangkrai Lerdthusenee; Ms. Ratana Sithiprasasna

b. Objectives:

1. Evaluate new repellent compounds against mosquito vectors of malaria and dengue using the human-skin bioassay. Determine ED50 and ED95 concentrations of each

repellent against selected mosquito species (Anopheles dirus, An. minimus, An. sawadwongporni, Aedes aegypti, and Ae. albopictus).

2. Evaluate new repellent compounds against chigger vectors of scrub typhus using an in vitro assay. Determine ED50 and ED95 concentrations of each repellent against selected chigger species (Leptotrombidium deliense and L. imphalum).

c. Methods:

1. We are able to evaluate up to 5 experimental compounds that have been approved for testing on human volunteers. Deet will serve as the appropriate reference compound. ED50 and ED95 concentrations of each repellent is determined against each of 5 selected mosquito species (Anopheles dirus, An. minimus, An. sawadwongporni, Aedes aegypti, and Ae. albopictus).

2. Chiggers: Determine ED50 and ED95 concentrations of experimental compounds provided by WRAIR against Leptotrombidium deliense and L. imphalum. Deet serves as the appropriate reference compound. Narrow bands of filter paper will each be treated with a given concentration of a given repellent, with control filter paper treated with ethanol only. A total of 4 concentrations of repellent are required in order to calculate the ED50/95 using a PROBIT Analysis Procedure. A band of repellent-treated filter paper is placed in a small plastic tube along with an individual chigger. The number of times that each chigger crossed the band is determined every minute for 10 mins, and ED50 and ED95 doses (dose that repelled 50% and 95% of chiggers, respectively) calculated.

d. Results (accomplishments during the period of January 2002 - December 2002):

Repellents evaluated against malaria & dengue vectors:
On human volunteers, Deet was superior to DM 159-2, DM 156-2, and DM 34-1 against Aedes albopictus.

Repellents evaluated against chiggers:
We have completed testing 4 additional repellent compounds (including DEET) against Leptotrombidium imphalum chiggers, using our newly developed rapid In Vitro procedure. The procedure requires only 5-mins and a small number of chiggers to obtain a valid estimate of the median effective dose (ED50). Our results indicated that DM159-2 repellent compound at the low concentration (1%) performed clearly better than the others two (DM156-d2 and DM34-1), but still inferior to DEET.

e Future plans:

We are funded by MIDRP to continue repellent testing in FY03.

3. Title of research project: Use of a Geographic Information System (GIS) to implement and evaluate the efficacy of targeted vector control as a means of reducing malaria transmission.

a. Investigators:

MAJ James W. Jones, PhD; Dr. Gabriela Zollner; Major R. Scott Miller MD; Benjawan Khuntirat, Ph.D.; Jetsumon Prachumsri, Ph.D.; Ms. Ratana Sithiprasasna

b. Objectives:

1. Identify key host and mosquito factors that affect the transmission of falciparum and vivax malaria in western Thailand,

2. Use these key factors to develop a Geographic Information System (GIS) that will predict malaria threat and can be used to develop targeted vector control strategies,

3. Implement a targeted vector control program within the study site and evaluate impact on malaria transmission.

4. Establish a well-characterized malaria study site that can be used to evaluate the proposed US Army Malaria Vector Control System.

5. A final component of this project is to conduct a field evaluation of dipstick assays for the detection of Plasmodium parasites in mosquitoes and in man.

c. Methods:

Information is being collected to construct Geographic Information System (GIS). The initial GIS was completed in 2002. Key components of the GIS include remote sensing data (vegetation and water sources), environmental (daily temperature, relative humidity, rainfall), demographic (house information, age, sex), parasitological (parasite species distribution, gametocyte rates, etc.), and entomological (adult and larval distribution and habitats as well as entomological inoculation rates) indices. In May-Jun 2002, cluster analysis was initiated to identify relationships between malaria cases and adult mosquito distribution, and between adult mosquito distribution and larval distribution. The GIS is being used to identify key sites for intervention (larval and adult mosquito control, as well as treatment of malaria reservoirs with transmission-blocking antimalarials). The different grids in the town will then be randomly selected as control or treatment sites. A maximum of 20% of known larval habitat, a maximum of 20% of houses, and a maximum of 20% of

d. Results (accomplishments during the period of January 2002 - December 2002):

1. Site Mapping: IKONOS (1 meter resolution) and LANDSAT (30 meter resolution) images of the village and surrounding area have been acquired and used to establish the GIS using ERDAS software.

2. Adult Mosquito Collections: The geographic and temporal distribution of each species is mapped out as collections are made. A total of 16,076 adult anophelines representing 37 species have been collected during landing/biting collections. A total of 11,007 mosquitoes have been tested by ELISA, with 22 positive mosquitoes.

3. Larval Mosquito Collection: To date (Jun 99-Mar 02), a total of 8,921 larval Anopheles mosquitoes representing 32 species have been collected, reared to adults, and identified. Identification of larvae through September 2002 continues.

4. Human Malaria Surveillance: To date (May 00-July 02), a total of 10,850 blood films have been collected from 646 individuals enrolled in the study.

5. Establishment of the GIS and malaria modeling: Mapping out of the village (houses, road, buildings, rivers) and larval mosquito habitats has been completed. A RadarSAT image has been registered to a LandSAT image and incorporated into the GIS to display the village in 3D fashion. Efforts to incorporate data (adult and larval mosquito collections, # of people per house, blood films collected, positive blood films, vegetation, etc.) into the GIS are underway.

6. Implementation of Malaria Control: Current effort has focused on evaluating the impact of the Thai Ministry of Public Health efforts. Our analysis is continuing.

e. Future plans:

Continue with study during FY03.

4. Title of research project: Development of a chigger-challenge model for the evaluation of candidate scrub typhus vaccines.

a. Investigators:

MAJ James W. Jones, PhD; Dr. Kriangkrai Lerdthusenee

b. Objectives:

1. Conduct genetic characterization of O. tsutsugamushi infecting 12 colonies of Leptotrombidium chiggers sps. maintained at AFRIMS.

2. Evaluate the ability of each of the 12 chigger colonies to transmit O. tsutsugamushi to laboratory mice. Down-select 4-5 key chigger colonies for further studies.

These chigger colonies should be infected with different strains of O. tsutsugamushi and should produce consistent, high infection rates when fed on mice.

3. Focus efforts on building up down-selected chigger colonies to the high levels required for potential vaccine studies.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Criteria used to assess efficacy must include quantification of rickettsemia in the mice; however, additional methods (clinical or immunological responses) may also be assessed.

c. Methods:

1. Characterization of Strains/Isolates of Orientia tsutsugamushi: Initially we will evaluate O. tsutsugamushi response to antibiotic agents and growth rates in a mouse model. We will also use heteroduplex PCR and gene sequencing of the r56 and 16SrDNA genes to characterize each of the strains.

2. Evaluate the efficacy of chigger colonies to transmit O. tsutsugamushi to mice and down-select 4-5 key colonies: Colonies of mites are being characterized by PFGE and DNA amplified fingerprinting to identify colony lines with the highest rate of infectivity to laboratory mice. Finally, we will attempt to characterize mechanisms by which Leptotrombidium deliense and L. chiangraiensis become infected with O. tsutsugamushi, to include analysis of vertical (mite to mite) and horizontal (vertebrate to mite to vertebrate) transmission. The ability to infect mites from infected hosts would allow us to evaluate the ability of potential vaccine candidates to prevent the transmission of O. tsutsugamushi from mites to vertebrates and subsequently back into mites.

3. Rearing key chigger colonies to levels sufficient to support vaccine challenge studies.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Initial efforts have focused on determining the course of rickettsemia over time for the 4-5 strains of O. tsutsugamushi selected for further study and on the development and/or confirmation of diagnostic procedures (PCR, ELISA, etc.) to quantify rickettsemia in challenged mice. Current effort is focused on use of TAQ-Man PCR. We also evaluated the effect of chigger infection with specific strains of O. tsutsugamushi on potential indicators of immunity, to include lymphocyte transformation, morbidity, and mortality.

d. Results (accomplishments during the period of January 2002 - December 2002):

We have conducted experiments to evaluate the infection rate and transmission efficacy of each of the 12 chigger colonies {one colony of L. deliense (Ld-Lines), 5

colonies of L. chiangraiensis (Lc-Lines), and 7 colonies of L.imphalum (Li-Lines)}, to transmit O. tsutsugamushi to laboratory mice. We successfully isolated 5 O. tsutsugamushi strains to the L-929 (mouse fibroblast) cells as followed. With the restriction fragment length polymorphism (RFLP) technique, we analyzed the 56kD major surface protein gene (corresponding to nucleotides 121-775 of the Karp strain) of this 5 isolates using 3 different enzyme digestions.. Our work associating clinical responses of laboratory mice to infection such as temperature, weight loss, and weight gain with the quantification of rickettsemia is essentially complete. Laboratory mice were infected with O. tsutsugamushi through feeding individual and pooled (2, 3 or more) scrub typhus-infected chiggers on the mice. Results demonstrated that infection rates from the feeding of all 3 Leptotrombidium species were high in both individual and pooled feeding. Infection of O. tsutsugamushi in L. chiangraiensis exhibited the highest rates in both individual and pooled feeding among all 3 species and also showed the most infection strength (determined by the total number of mice dying earliest) as followed: 50% on the 12th day and 41.8% death rate on the 13th day after infection in the individual and pooled feeding, respectively.

e. Future plans:

Work is continuing under MIDRP funding

5. Title of research project: Optimization of sporozoite production to support a human Plasmodium vivax sporozoite-challenge model.

a. Investigators:

MAJ James W. Jones, PhD.; Jetsumon Prachumsri, Ph.D.; LTC Robert Scott Miller.

b. Objectives:

To develop methods of producing mosquitoes with consistent, reproducible salivary gland infections that will minimize variation in the course of human infection following sporozoite challenge.

c. Methods:

1. Basic Sporozoite Challenge System : We initially worked to establish a "basic system" to provide sporozoite-infected mosquitoes in support of STEP/STO requirements.

2. Refined Sporozoite Challenge System: Once our "basic system," was established subsequent efforts focus have been to refine the system in order to reduce the variability in the mosquito infections (critical for ensuring consistent challenges) and to eliminate the risk of concomitant mosquito infections. The goal is to develop a system that will i) consistently provide mosquito infection rates with >60% of blood-fed mosquitoes having +3/4 (> 100 sporozoites) salivary gland infections, and ii) provide P. vivax-infected mosquitoes that do

not harbor concomitant pathogens

Plasmodium vivax-infected patients reporting to local malaria clinics serve as the starting point for development of the "refined system." Instead of allowing mosquitoes to feed directly on gametocytemic patients, mosquitoes are fed on venous blood provided to them in an artificial membrane feeding system. A series of carefully controlled experiments are conducted using patient blood and the membrane feeding system. Each of these experiments provides "stand-alone" data to support the development of the sporozoite-challenge model; however, each experiment will build upon the information derived from the previous experiment. The following are areas of focus:

a. Accomplish dilution of Blood to a standard Gametocyte Concentration
b. Replacement of Patient Sera with Commercial Sera (The reconstituted blood will be fed to mosquitoes in a membrane feeding system and mosquito infections quantified. This method has the advantage of removing anti-malaria antibody that may affect gametocyte infectivity)
c. Use of Frozen Gametocyte Preparations to infect Mosquitoes

d. Results (accomplishments during the period of January 2002 - December 2002):

1. New SOP developed for membrane feeding which gives more consistency of the mosquito feeding rate.

2. Replacement of patient plasma with pooled AB serum from malaria naïve donors increased infectivity in mosquitoes. There will be more experiments to compare serum from different blood group besides AB.

3. Short-term culture of P. vivax parasites (from 6 to 48 hrs) increased the parasite infectivity to the mosquitoes. For some cases after culturing the infective rate was increased from 0 to 80%.

4. Blood from thirty cases of P. vivax infected patients was collected at the Mae Sod malaria clinic and cryopreserved. Blood from sixteen of those cases was thawed and put into culture at the Bangkok laboratory. Over half the cases were kept in culture for over 30 days. Parasitaemia also increased after culture. After 1 month of culture the packed cell volume also increased. During long-term culture, gametocytes were produced but minimal mosquito feeding has been accomplished.

5. We hope to compare growth of P. vivax parasites when different sources of reticulocytes are used for long-term culture.

e. Future plans:

Continue in FY03.

B. Department of Immunology AFRIMS FY02 Research Accomplishments

1. Title of research project:

Number	Projects	Status
1	MRDD Phase III (687-2001)	In life completed; Analysis
2	Human Malaria Vivax Challenge	Protocol dev't
3	In Vivo Efficacy Sangkhla	In progress
4	Microscopy QA	In progress
5	Mefloquine Resistance Genetics	In progress
6	Rickettsial Isolation	In progress
7	Phase II Azithro-quinine	In life Complete; Analysis
8	Phase I/II TQ Radical Cure	delay; protocol development
9	Fever II Sangkhla	Starting Feb 02
10	Fever I Sangkhla	In life completed; analysis
11	TQ Radical Cure Part II	manuscript
12	Flavivirus Surveillance	In progress
13	Nepal Microscopy QA/Filter	Protocol Devt
14	MRDD Phase II (818)	Completed; manuscript
15	Binax MRDD -treatment	Completed; analysis
16	In Vitro Sensitivity Techniques	Completed; PhD thesis
17	Bangladesh In Vitro Pf	Paper Submitted
18	Leptospirosis	Manuscript
19	MRDD - others	summer
20	Isolates Myanmar	August
21	Isolates Vietnam	September
22	Phase I/II TQ Pediatric	delay to at least FY03
23	Azithro/Artesunate	Delay at NIH; Pfizer to FY02
24	Rhesus AL/AS Efficacy	In progress
25	Rhesus AL/AS Toxicology	IACUC
26	Rhesus AL/AS PK/PD	In progress
27	Febrifugine	In progress
28	TQ Measurement - Phase II	data analysis
29	Bioassay/HPLC Validation - FDA	AS complete; In progress AL
30	Plasmepsin/MAK Kinases	In progress
31	Biguanide Bioassay	Samples collected
32	Gametocyte Production	In Progress
33	IV AS PK	In Progress
34	Mitochondria Tox	in progress
35	P. cynomolgi/ relapsing malaria	to start in August
36	Mouse EE model	update??

Number	Projects	Status
37	HE2000 Bioassay	Protocol devt
38	Rhesus Pf AMA-1 Vaccine	IACUC
39	RTS,S/TRAP Neonates	Report due
40	ELISPOT Automated Reader Validation	In progress
41	MSP-1 and Innate Immune cells	in progress
42	PvDBP Polymorphisms	PhD project
43	Human Dendritic Cell /Ag competition	Protocol Devt
44	MSP1-19 with CPG	Protocol Devt
45	Better Th1 Responses in Mice	In progress
46	Rhesus PfMSP-1 Vaccine	completed; paper in progress
47	MSP-1 and complement	In progress
48	Dengue activates plasmacytoid DCs	In progress
49	Schizont activates plasmacytoid DCs	In progress
50	Role of Antigen presenting cells/□□T cells	In progress
51	Rhesus/CgGODN	manuscritp submitted
52	PCR for MRDD	in progress
53	Khun Anintita's PhD	
54	Vivax Genotyping - Kong Mong Tha	in progress; transition Ento
55	Vivax Genotyping - PV Mahidol	On hold
56	Folate Resistance- Nepal	Contract Kenya
57	Scrub Typhus Dipstick	In progress

a. Investigators:

Dr. R. Scott Miller, Dr. Suping Jiang, Dr. Chansuda Wonsrichanalai, Dr. Sathit Pichyangkul, Dr. Paktiya Teja-Isavadharm, Dr. Harald Noedl

b. Objectives:

To protect, project and sustain the military soldier against disease threats produced by the 2 major species of malaria, *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv). To support this mission through the evaluation of new or improved vaccines, prophylactic and therapeutic drugs, rapid diagnostic kits, and the maintenance of a center for excellence focused on the basic biology and epidemiology of malaria. Secondly, to assess emerging febrile diseases along high-risk regions of SE Asia, particularly the Thai-Myanmar borders.

c. Methods:

The department of Immunology and medicine has applied as many kinds of classical and state-of-the-art technologies as possible to above multi-facets researches. The detail methods for the clinic and pre-clinical research include field sample collection and screening,

microscopy, clinic diagnosis, patient treatment, laboratory animal test and observation. The techniques used for the mechanism studies of vaccine and drugs include advanced molecular biology and flow cytometry technologies, HPLC, bioassay, cell culture, biochemical analysis and so forth.

d. Results (accomplishments during the period of January 2002 - December 2002):

1. Malaria Vaccines STEP F/STO AF/STO A1

- Initiated development of a complex, multi-year project to develop a *P. vivax* human malaria challenge model. This model will allow better understanding of immunologic processes early in parasitemia, phase IIa pilot efficacy studies of candidate Pv vaccines, and testing of drugs with causal prophylactic and radical cure properties of Pv in a controlled setting. This project is partnered with the Vaccine Trial Center, Faculty of Tropical Medicine, Mahidol University. Efforts continuing in to FY03 to complete the validation of the model. MIDRP funded.
- Completed the in-life portions of a rhesus safety and immunogenicity protocol for a candidate Pf malaria multi-antigen, multi-stage vaccine. The vaccine involves combinations of RTS,S, $MSP1_{42,}$ and AMA-1 candidate vaccines, adjuvanted with our lead agent, AS02A. 46 rhesus monkeys were immunized over a 3-month schedule, and the vaccine is safe and well tolerated. Antibody and cellular immunology assays are in progress, and data analysis will be completed in FY03. This MIDRP effort is partnered with GlaxoSmithKline and USAID.
- Completed evaluation of candidate Pf vaccine, $MSP1_{42}$, in rhesus, which is now in early phase human studies. MIDRP/USAID funded. Publication submitted.
- Continued assessment of safety and immunogenicity of RTS,S/TRAP vaccine candidate in neonatal rhesus monkeys. GSK and WHO funded. Publication is pending.
- Investigated interplay of MSP-1 with the innate immune system and how this may cause aspects of severe malaria. The 33-kD component of MSP-1, which is cleaved into the blood stream upon parasite entry in the red cell, is a potent trigger of the innate immune system. Impact on vaccine development is being investigated, and this may be a target in prevention of severe malaria. ILIR funded.
- Continued efforts to incorporate ways to enhance immune responses in mice using novel adjuvant systems (CpG ODNs) and other novel adjuvant systems. ILIR and now MIDRP funded.

2. Malaria Drugs STEP Q, STO-AQ, STO-A4, STO-A5

- Executed complex studies of a severe malaria model using *P. coatneyi* in splenectomized rhesus for efficacy testing of candidate intravenous artemisinins (lead agents for the new drug to replace quinidine). Presented for down-selection meeting in October 2002 and artesunate was selected as lead compound. Report in progress. This MIDRP effort is partnered with the Medecins for Malaria Venture (MMV).
- Completed the development and in-life phase of testing intravenous artemisinin candidates (artelinate and artesunate) head-to-head in standard toxicology protocol

(including neurotoxicity studies) in rhesus. Preliminary analysis completed and presented at down selection meeting in October 2002. The data was critical to the down-selection of artesunate as the lead agent for clinical development. Analysis of brains for subtle neuropathologic changes is ongoing, but critical for pre-IND meeting with the FDA in March 2003. MIDRP and MMV funded.

- Adapted and completed in vitro bioassay and HPLC-ECD of rhesus samples to allow measurement of total antimalarial activity of intravenous artemisinins as well as measurement of major metabolites, in support of rhesus efficacy and toxicity trials. Presented at down selection meeting in October 2002. GLP validation in progress for planned Phase I human studies in FY04. MIDRP and MMV funded.
- Presented data from the Phase II dose ranging trial of azithromycin/quinine combinations for the treatment of uncomplicated falciparum malaria. This was the first clinical trial completed in the new AFRIMS clinical trial center co-established with Kwai River Christian Hospital in Sangkhlaburi along the Thai-Myanmar border. This has spawned renewed interest in azithromycin as an antimalarial, and prompted further studies in both Africa and Thailand. Efforts are partnered with Pfizer and the NIH.
- Developed a program to test tafenoquine (WR238605) in adults and children for evaluation of radical curative ability and pharmacokinetics in *P. vivax* malaria. Funded with NIH co-development grant with GSK, and partnered with Hospital of Tropical Diseases, Faculty of Tropical Medicine, Mahidol University. Phase II trial delayed to FY03. Publication of previous dose-ranging studies of tafenoquine for prophylaxis and radical cure are in progress.
- Screened derivates of the febrifugine for antimalarial activity and toxicity in mouse models and cytotoxicity assays. Febrifugines are candidate drugs in the discovery phase of development, extracted from the Chinese herb, Chang Shan (*Dichroa febrifuga* Lour)
- Further screening and toxicity of plasmepsin- and diphenylurea-based antimalarial lead compounds. Lack of suitable candidate led to decision to suspend further development.
- Developed a model for assessing toxicity in neuronal cell lines, which will require validation in FY03. Efforts at *Plasmodium* mitochondrial toxicity assays were unsuccessful. MIDRP funded.
- Supported continuing efforts to develop a hepatocyte cell line to screen activity in the liver of antimalarial drugs and vaccine candidates. Collaboration with Department of Entomology. MIDRP funded.

3. Diagnostics/Rapid Diagnosis of Malaria STEP-L/STO-L

- Completed the in-life and microscopic assessment portions of the definitive multi-center trial of a second-generation malaria rapid diagnostic kit (NOW ICT Pf/Pv) in Mae Sod (n=2400), in association with WRAIR, NAMRID Peru, USAMMDA and Binax Inc. Now in final data analysis. A final bridging study before FDA licensure is planned in FY03.
- Evaluated 3 commercial and 1 prototype malaria rapid diagnostic tests (MRDDs) for performance characteristics in the field. Completed 3 publications of previous tested MRDDs.

- Evaluated two methods of real-time PCR to diagnose malaria, especially mixed infections, which are difficult to detect by microscopy. Supported a blinded comparison of these methods to expert microscopy in coordination with Department of Enterics/AFRIMS and Department of Immunology at WRAIR. Method now in standard use. MIDRP funded.

- Evaluated available scrub typhus tests to develop a gold standard for comparison to immunochromatographic scrub typhus (*Orientia tsutsugamushi*) rapid diagnostic tests in development. Helped submit an SBIR, that is funded for FY03, to spur further development of a rapid diagnostic assay.

4. Emerging Infectious Diseases (GEIS)

- Epidemiology of Falciparum Malaria Drug Resistance Patterns in Asia:

Continued surveillance activities throughout Southeast Asia (Bangladesh, Myanmar, Thailand and Vietnam) for threat assessment of multi-drug resistant malaria. Looked at new field sites in Sri Lanka, Nepal and northern Thailand (Chiang Dao and Mae Hong Sorn). No emergence of artemesisin resistance has been detected, but evidence that mefloquine resistance has spread across Myanmar and into Bangladesh. Data presented innumerous forums and samples archived in the cryobank for further analysis as needed. GEIS funded, and coordinated with Public Health departments in the various countries.

- Completed an *in vivo* trial of efficacy of mefloquine along a high-risk area of the Thai-Myanmar border, Sangkhlaburi district. Results reveled mefloquine failure rates of 45%, prompting the government to change the treatment paradigms in this region. A follow-on study of the new regimen (mefloquine and artesunate) has revealed 100% efficacy. Funded by GEIS and an NIH R01 grant with University of North Carolina.

- Compared the clinical responses of the above trial to in vitro drug testing and genetic markers to show a very high correlation of select polymorphisms with mefloquine resistance. These associations are by far the strongest yet reported and confirm the role of pfMDR-1 mutations in mefloquine resistance. Funded by NIH grant with University of North Carolina.

- Developed and validated a new non-isotopic method for *in vitro* drug resistance assays, which is simpler, as robust, and avoids radioisotopes. The methods has been made available free of charge to the malaria research community as a public service (see http:/malaria.farch.net). Field validations are in progress. Funded by GEIS with support from Mahidol University and University of Vienna.

- Developed and executed a fever surveillance trial in the southeastern Terai region of Nepal with the objectives to ascertain the rates of malaria and assess Pf isolates for the severity of sulfadoxine pyrimethamine resistance by molecular methods. In life portion successfully completed despite the civil war in September 2002. The malaria rates are 3 times higher than reported locally, largely due to poor quality malaria diagnostics/microscopy. Genetic analysis is ongoing. Training seminars in malaria diagnosis were held in the Vector Borne Diseases Research and Training Center, Hetauda Nepal. Funded by USAID and GEIS.

- Surveillance of Febrile Diseases along the Thai-Myanmar Border:

- Completed a multi-year effort to establish infectious etiologies to undifferentiated fevers along the Thai-Myanmar border in Kanchanaburi province. Over 850 persons enrolled. Malaria accounts for approximately 25% of adults with fever. Leptospirosis appears to be a frequent, but previously unrecognized cause of morbidity and mortality, as is spotted fever rickettsia. Our data has led to a change to local health treatments.
- Serological evidence two Spotted Fever fever rickettsias not described in Thailand has been made. Prospective study for rickettesial diseases including rodent isolation techniques has not yet yielded a pathogen. Tick and flea collection have yielded numerous new *Rickettsia,* and *Ehrlichia* organisms of unknown pathogenic potential.
- Serologic studies suggest an unusual flavivirus infection in this population. Mosquito collections have reveled extensive local Tembusu virus transmission, and unusual JE-like viruses currently under evaluation at USAMRIID. Joint effort with Entomology, Vet Med and USAMRIID.

e. Future plans:

We plan to continue our multi-faceted emphasis on support for malaria product development in diagnostics, new drugs, and new vaccines. We anticipate involvement in the malaria rapid diagnostic testing until eventual US FDA licensure, and development of a DoD wide effort on malaria microscopy QA procedures. Furthermore, we anticipate being the lead overseas lab for field-testing intravenous artesunate in phase I and II, as possibly phase III testing. We will continue efforts for tafenoquine development, especially towards an indication of radical cure for *Plasmodium vivax*. We will continue safety and immunogenicity testing of candidate malaria vaccines in rhesus, and progress towards vivax challenge studies for eventual human testing of vivax vaccines in Thailand. Emerging infection work in Sangkhlaburi will continue with emphasis on flaviviruses, leptospirosis and rickettsial illnesses, and this study may be expanded to another targeted site in SE Asia. Lastly, we anticipate an expand role in regional malaria surveillance with a combination of in vivo, in vitro and genetic methods to define expanding malaria drug resistance.

C. Department of Enteric Diseases, AFRIMS FY02 Research Efforts.

1. Title of research project: Surveillance of Diarrheal Diseases in Adult Travelers and Residents from Developed countries and Thai Adults: Etiology and Antibiotic Susceptibility Pattern Transmission-dynamics of anti-biotic resistant scrub typhus.

a. Investigators:

Sinn Anuras, MD; Carl Mason, COL ; Ladaporn Bodhidatta, MD

b. Objectives:

To describe the etiology of acute diarrhea in travelers, expatriates and Thai adults and the antibiotic susceptibility pattern of enteric pathogens.

c. Methods:

Cases with diarrhea and asymptomatic controls were enrolled from Bumrungrad hospital. Demographic data was completed. Stool specimens were collected for microscopic examination, stool culture for enteric pathogens, ELISA test for rotavirus, Giardia and cryptosporidium. Enteric pathogens were tested for susceptibility to commonly used antibiotic by disk diffusion method.

d. Results:

418 of travelers/expatriates with acute diarrhea and 418 asymptomatic controls were enrolled. Enteric pathogens that were significantly isolated from cases than from asymptomatic controls were Campylobacter (14%VS 1%), V.parahemolyticus (13% VS 0.5%) and ETEC (8% VS 3%). Campylobacter spp isolated from cases were highly resistant to ciprofloxacin (90%) but still sensitive to azithromycin. Ciprofloxacin is still effective against other pathogens.

Among Thai adults, 400 cases of diarrhea and the same number of controls were participated. Rate of pathogen recovery was significantly lower than in the foreigners (42% VS 53%). V.parahemolyticus and ETEC were significantly detected among cases than among controls (10% VS 0.5% and 5% VS 1%). Salmonella and Campylobacter were isolated from cases in the same number as from controls.

Part of the data was presented at the 102nd meeting of American Society of Microbiology, Salt Lake City, Utah, USA in May 2002.

e. Future plans:

Completion of the study. Data will be analyzed and a manuscript will be prepared after the completion of laboratory tests.

2. Title of research project: Surveillance of Diarrheal Diseases in Travelers and Resident Expatriates in Nepal: Etiology and Antibiotic Susceptibility Pattern.

a. Investigators:

Surveillance of Diarrheal Diseases in Travelers and Resident Expatriates in Nepal: Etiology and Antibiotic Susceptibility Pattern

b. Objectives:

To describe the etiology of acute diarrhea in travelers, expatriates in Nepal and the antibiotic susceptibility pattern of enteric pathogens.

c. Methods:

Cases with diarrhea and asymptomatic controls were enrolled from CIWEC clinic, Kathmandu, Nepal. Demographic data was completed. Stool specimens were collected for microscopic examination, stool culture for enteric pathogens, ELISA test for rotavirus, Giardia and cryptosporidium. Stool culture and identification was initially performed in CIWEC clinic and the isolates were subsequently confirmed by AFRIMS.

Enteric pathogens were tested for susceptibility to commonly used antibiotic by disk diffusion method.

d. Results:

Among patients with diarrhea, 140(44%) were resident expatriates and 180 (56%) were tourists. Of 156 controls, 97(62%) were residents and 59 (38%) were tourists. Enteric pathogens were identified in 70% of cases and 26% of controls. Primary enteric pathogens for tourist cases were *Campylobacter* (20%), *Shigella* (20%), *ETEC* (14%), *Rotavirus* (13%), *Giardia* (11%), *Aeromonas* (10%), *Cyclospora* (5%) and *EAE* (5%). For resident expatriates, the enteric pathogens were *Cyclospora* (16%), *Campylobacter* (14%), *ETEC* (11%), *Giardia* (11%), *Aeromonas* (11%), *Rota virus* (10%), *Shigella* (6%) and *EAE* (4%). *Cyclospora* was found only in cases and not among controls. Of 50 *Campylobacter* isolates 59% were resistant, 7% had intermediate sensitivity, and 33% were sensitive to ciprofloxacin. One hundred percent of *Campylobacter* isolates were sensitive to azithromycin. One hundred percent of *Shigella* isolates were sensitive to ciprofloxacin. *Shigella* sensitivity to azithromycin was 29% intermediately sensitive and 71% fully sensitive. *E.coli* isolates were 98% sensitive to ciprofloxacin, and 78% sensitive, 18% intermediately sensitive and 4% resistant to azithromycin.

e. Future plans:

Enrollment will be continued until March,2003 After completion of the study, data will be analyzed and a manuscript will be prepared.

3. Title of research project: Characterization of Enteric Pathogens isolated from Children in Hanoi.

a. Investigators:

Phung Dac Cam, MD; Ladaporn Bodhidatta, MD; Carl Mason, COL

b. Objectives:

1. To describe the prevalence of enteric pathogens in Vietnamese children with acute diarrhea and matched controls in Hanoi.
2. To describe the antibiotic susceptibility pattern of enteric pathogens.

c. Methods:

A year- long prospective surveillance was conducted. Children under 5 years of age with diarrhea and matched controls were enrolled from Saint Paul Hospital, Hanoi, Vietnam. Demographic data was completed. Stool specimens were collected for microscopic examination, stool culture for enteric pathogens, ELISA test for enteric viruses (rotavirus, adenovirus and astrovirus), Giardia and cryptosporidium. Stool culture and identification was initially performed by the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi and the isolates were subsequently confirmed by AFRIMS. Enteric pathogens were tested for susceptibility to commonly used antibiotic by disk diffusion method.

d. Results:

A total of 291 cases and 291 controls were enrolled in the study. *Shigella* , *Salmonella*, *Campylobacter* and ETEC was detected in 8%, 6%, 4% and 3%, respectively. Isolation rate of bacterial enteric pathogens in controls was significantly lower than in cases. Rotavirus was significantly found in cases comparing to controls (47% VS. 25%, $p<0.0001$). Astrovirus was identified in 12% of cases and 1% of controls ($p=0.001$) while adenovirus was detected in 4% of cases and 1% of controls. Quinolone is generally an effective drug for treatment of bacterial diarrhea but emergence of quinolone resistant campylobacter was observed in 36%. Campylobacter isolates are still sensitive to macrolides.

Data will be presented at the 103rd meeting of American Society of Microbiology, Washington DC, USA in May 2003.

e. Future plans:

Completion of the study. Data will be analyzed and a manuscript will be prepared after the completion of laboratory tests.

4. Title of research project: Case-Control Study of Endemic Diarrhea in Children in Sangkhlaburi; along the Thai-Myanmar border.

a. Investigators:

Ladaporn Bodhidatta, MD; Philip McDaniel, MD; Carl Mason, COL

b. Objectives:

1. To describe the prevalence of enteric pathogens in Thai children with acute diarrhea and matched controls in Sankhlaburi.
2. To describe the antibiotic susceptibility pattern of enteric pathogens.

c. Methods:

A field laboratory was set up and a year- long prospective surveillance was conducted. Children under 5 years of age with diarrhea and matched controls were enrolled from Kwai River Christian Hospital, Sankhlaburi, Thailand. Demographic data was completed. Stool specimens were collected for microscopic examination, stool culture for enteric pathogens, ELISA test for enteric viruses (rotavirus, adenovirus and astrovirus), Giardia and cryptosporidium. Enteric pathogens were tested for susceptibility to commonly used antibiotic by disk diffusion method.

d. Results:

A total of 236 cases and 236 controls were enrolled. Campylobacter, Enterotoxigenic E.coli (ETEC), Shigella and Salmonella were isolated from 21%, 10%, 8% and 6% of cases and 24%, 7%, 0.5% and 8% of asymptomatic controls, respectively. Rotavirus was detected in 18 % of cases and 3% of controls. Giardia was frequently identified among cases as well as controls in 13% and 22%, respectively.
Enterotoxigenic E.coli (ETEC), Shigella and Salmonella isolates were uniformly sensitive to ciprofloxacin but fluoroquinolone resistant was detected in 40% of Campylobacter isolates.

e. Future plans:

Completion of the study. Data will be analyzed and a manuscript will be prepared after the completion of laboratory tests.

5. Title of research project: Development and standardization of realtime PCR assays for detection and characterization of enteric pathogens.

a. Investigators:

Orntipa Sethabutr, Carl Mason, COL

b. Objectives:

1. To develop, standardize and evaluate realtime PCR assays for qualitative/quantitative detection of Shigella/EIEC, Salmonella, Campylobacter and Cyclospora.

2. To establish Standard Operating Procedures for routine detection and characterization of Shigella/EIEC, Salmonella, Campylobacter and Cyclospora.

c. Methods:

1. Sequences of gene targets of enteric pathogens of interests were searched from public nucleotide sequence databases, BLAST alignment was performed for homology information and primers, probes were designed by Primer Express Software.

2. Primers and probes were synthesized by oligonucleotide synthesis facility that meets good manufacturing practice (GMP) standards. Probes were labeled with 5'-FAM as a fluorescent reporter and with 3'-TAMRA as a quencher.

3. DNA templates for PCR were prepared from bacterial cultures and stool samples by simple boiling in Tris-EDTA-detergent buffer. In case that PCR inhibitors needed to be removed, samples were subjected to DNA purification using QAIGEN stool kits.

4. The reaction mixture of realtime PCR consisted of 2 μL of DNA template, 1X TaqMan bufferA (PE Biosystem), 2mM $MgCl_2$, 100 nM each of dNTPs, 200 nM of primers, 20 nM of fluorescently-labeled probe and 2.5 unit of AmpliTaq Gold in 50 μL of total reaction volume.

5. The realtime PCR assays were conducted using ABI7700 or ABI7000 Sequence Detection System. Amplification profiles consisted of enzyme activation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 30 seconds, and of primers annealing, extension and probes hybridization at 60°C for 1 minute.

d. Results, Future plans and other details:

Shown in table 1.

Table 1: Realtime PCR detection systems for enteric pathogens identification developed and tested during the period of January – December 2002.

Pathogens	Target Genes	Accomplishments	Future Plans
Shigella and EIEC	Invasion plasmid antigen H (ipaH). Primers and probes designed by Huo-Shu Houng.	1. Completed evaluation against standard reference strains. 2. Completed evaluation in clinical stool samples (n=400). 3. Established SOP for qualitative detection.	- Use in CG03. - Use with IVI samples. - Use as alternative method to identify Shigella and EIEC from clinical samples - Develop quantitative detection.
Salmonella	Flagellin gene (fla) specific for *Salmonella spp.*	1. Designed primers and probes. 2. Completed evaluation against known reference strains.	- Continue evaluation in stool samples.
	Virulence gene (viaB) specific for *S. typhimurium*	1. Designed primers and probes. 2. Completed evaluation against known reference strains.	- Continue evaluation in stool samples.
Cyclospora	Small subunit ribosomal RNA genes	1. Designed primers and probes. 2. Completed evaluation against known reference strains.	- Continue evaluation in stool and environmental samples.
Campylobacters	ceuE gene specific for C. coli and C. jejuni. Primers and probes designed by Huo-Shu Houng.	1. Completed evaluation against known reference strains. 2. Completed evaluation against clinical stool samples (n=350). 3. Established SOP for qualitative and quantitative detection.	- Use in CG03. - Use as alternative method to identify CJ and CC from clinical samples.
	glyA gene specific for C. upsaliensis and C. lari	- Designed primers and probes. - Completed standardization of PCR conditions.	- Continue evaluation against known reference strains - Continue evaluation in stool samples.

6. Title of research project: Amplified Fragment Length Polymorphism (AFLP) fingerprint method to characterize enterotoxigenic E. coli (ETEC) colonization factors (CFs) and to study the genetic relationship among serotypes of Shigella flexneri.

a. Investigators:

Orntipa Sethabutr, MS.; Carl Mason, COL

b. Objectives:

To investigate the genetic relationship between AFLP fingerprints patterns and the defined phenotypes of ETEC based on colonization factors. To establish molecular methods to identify potential variants of ETEC carrying uncharacterized colonization factors. To investigate the genetic relationship among different serotypes of *Shigella flexneri.* To construct a database of AFLP fingerprint profiles corresponding to each serotype that can be applied for categorizing untypable strains.

c. Methods:

1. Bacterial isolates selected for studies (known and unknown) were grown in specific media, cells were harvested and subjected to genomic DNA purification using standard phenol-chloroform extraction followed by alcohol precipitation. 2. Purified DNA was digested with restriction enzymes, MseI and EcoRI, ligated to specific adapters before pre-selective and selective amplification reaction conducted. The amplified products were separated by capillary gel electrophoresis on an ABI310 Automated Genetic Analyzer (Applied Biosystem, CA). Data obtained between 50 and 500 basepairs were processed and calculated into binary scoring format by using Genotyper Software (Applied Biosystem) with peak height setting at 150 unit and plus/minus 0.5 bp tolerence. DNA fingerprints data were compared and grouped in correlation with the types of colonization factor and toxin expression of ETEC. In case of *Shigella flexneri*, fingerprints data were grouped in correlation with known serotypes. Cluster analysis was conducted using Bionumerics Software (Applied Maths, Belgium).

d. Results-ETEC:

Ninety-eight ETEC strains isolated from 3 sites in Thailand were primarily selected for ETEC study. The strains were subjected to confirmation of toxin determinants (LT, STIa and STIb) by conventional multiplex PCR and to confirmation of CFs by monoclonal antibody dot-blotted ELISA.

e. Results-Shigella:

Banding profiles of *S. flexneri* based on serotypes 1, 2a, 3a, 4 and 6 were clearly distinguished. Similarity analysis demonstrated the similarity between individual profiles ranging from 78-98% within each serotypes. The genetic relationships among serotypes 1, 2a, 3

and 4 were closely related while the serotype 6 was separately clustered. Standard AFLP using three selective primer sets of EcoRI and MseI allows a sufficient discrimination between different serotypes and strains of S. flexneri. The unique banding profiles of each serotype can be applied as a database for categorizing untypable strains.

f. Future plans-ETEC:

We will continue to investigate at least 5 ETEC isolates per known CF including new variants of CF from our collaborators. Examination of a group of ETEC isolates with unknown CFs and analysis of combined AFLP and immunological data will be performed.

g. Future plans-Shigella:

Approximately 50 Shigella flexneri isolates collected from areas outside Thailand will be included in this study and the AFLP profiles as well as cluster analysis will be compared with our database.

7. Title of research project: ***Mechanisms of Antibiotic Resistance in Campylobacter jejuni***

a. Investigators:

Warawadee Nirdnoy, MS.; Orntipa Sethabutr, MS.; Carl Mason, COL

b. Objectives:

1. To determine the incidence of transposons and plasmid encoded antibiotic resistance genes on plasmids in selected CJ strains isolated from Thailand over a span of 15 years.
2. To study the relevant structure, repeat sequences and transposons in the plasmids of CJ strain #8245.

c. Methods:

CJ strain #8245 were inoculated onto blood agar plates and incubated at 37°Cfor 48 hours under microaerobic condition. Plasmid DNA of CJ were prepared using QAIGEN kits. Plasmid DNA were subsequently digested with restriction enzymes, cloned and subcloned into pBluescript cloning vector using *E. coli* DH5 alpha as transformant host. The transformants were selected on LB agar supplemented with ampicillin and X-gal to differentiate the recombinant DNA. The recombinant plasmids were subjected to DNA sequencing by Transposon insertion followed by Big Dye Terminator sequencing (Applied Biosystems,CA) and analysed on an ABI310 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using Sequencher 4.1 and Mac Vector. DNA and protein searched were performed using BLAST analyses via public NCBI database.

d. Results:

pproximately 20 kilobases (kb) fragment derived from CJ plasmid responsible for several antibiotic resistance phenotypes were cloned, and subcloned into pBluescript vector. So far, up to 8 Kb of cloned fragments were sequenced.

e. Future plans:

Continue to complete DNA sequencing of the 20 kb fragment.

8. Title of the project: Stablishment of a non-human primate Campylobacter disease model prior to the pre-clinical evaluation of Campylobacter vaccine formulations.

a. Investigators:

Michael Lewis, MAJ, MC; Daniel Scott, CAPT, USN MC (NMRC), Montip Gettayacamin, DVM; Carl J. Mason, COL, MC; Dilara Islam, PhD; Ladaporn Bodhidatta, MD; Shahida Baqar, PhD (NMRC).

b Objectives:

Establish a non-human primate model prior to pre-clinical evaluation of candidate vaccines to elicit protection against *Campylobacter* by documenting the infectivity of rhesus monkey and to evaluating the immune response elicited by a fully virulent *Campylobacter* strain.

c Methods:

Experimental Design: *Macaca mulatta* monkeys are being challenged with *Campylobacter jejuni* 81-176. Three groups of 10 monkeys are being used sequentially starting with challenging one group with the smallest dose (10^7), followed by challenging one group with 10^9 and another group (will be) with 10^11, to establish an attack rate of 80% or greater by Day 14 post-challenge. 5 monkeys are being used as control group.

Fecal excretion of *C. jejuni* are being monitored. Collected stool samples also being used to measure secretory IgA on days 0, 7, 14, 21 and 28.

7 ml of blood are being drawn on days 0, 7, 14, 28 for plasma for IgG, IgM, IgA titers (humoral immunity) and peripheral blood mononuclear cells (cell-mediated immunity) antibody secreting cell (ASC) assays.

Stool characteristics: Stool characteristics of all monkeys are being assessed twice per day according to the SOP.

Stool ASC: Antibody secreting cell assays are being performed on peripheral blood mononuclear cells against a glycine extract of *Campylobacter* outer surface preparation and formalin fixed whole cell *Campylobacter jejuni* 81-176.

ELISA: ELISAs are being performed using plasma samples and fecal extracts against a glycine extract and formalin fixed whole cell *Campylobacter jejuni* 81-176.

d Result :

This is an on going project. By another 3-4 months third challenge with 10^11 will be done and then data analysis will start.

e Future plans:

Based on the out comes of this project, another project titled" Preclinical trial of a Campylobacter Whole Cell (CWC) vaccine composed of formalin-inactivated Campylobacter jejuni, strain 81-176 in rhesus monkey model to assess efficacy, immunogenicity and safety" will be carried out on year 2004.

9. Title of the project: Travelers' Diarrhea Among US Forces Deployed for Operation Cobra Gold

a. Investigators:

Carl J. Mason, COL, MC; Ladaporn Bodhidatta, MD, Michael D. Lewis, MAJ, MC; Dilara Islam, PhD.

b. Objectives:

To determine incidence and etiology of travelers' diarrhea in US Forces deployed for CG'02. To determine prevalence on arrival and departure of antibodies to Campylobacter. To determine antimicrobial susceptibility patterns of enteric pathogens isolated.

c. Methods:

The target population was the approximately 1300 US soldiers deployed to Camp Nimnan Khollayoot, Sa Kaew, Thailand for CG'02. Cases with acute diarrhea and controls were required to complete a questionnaire, provided stool specimens for microbiology evaluation, and underwent phlebotomy twice (10 mL each time): once at enrollment and again 14 days later.

ELISA:

C.jejuni specific antibodies were screened by standard ELISA for IgA and IgG. Fecal extracts are evaluated for the presence of antigen specific secretory-IgA. Campylobacter specific serum IgA, IgG response and fecal s-IgA response will be able to define patients, asymptomatic carrier and negative control group.

Characterization of Surface Proteins and Immunoblot Assay:

Membrane proteins from the isolated Campylobacter strains will be compared with that of 81-176 strain by glycine extract procedure. By comparing the immunoblots of acute and convalescent patient fecal IgA extracts we will identify protein bands to which the patient is mounting a mucosal immune response. If these same protein bands are recognized by sIgA from asymptomatic carrier it is likely that they play a role in immunity to disease and perhaps, even infection. Campylobacter specific fecal IgA extracts can help to identify the most promising protein candidates for construction of effective vaccine.

Cellular Immune Responses:

Will be assessed by measuring cytokines in plasma and fecal extract. To determine whether Campylobacter infection derives Th1 or Th2 type cytokines production, plasma and fecal extract will be assayed for IL-2, IFN-g, IL-4, IL-5, IL-6, IL-8 and IL-10 by the FlowMetrix (TM) System (Luminex, Austin, TX).

d. Results:

Seventy-six cases of diarrhea were enrolled. Salmonella, Campylobacter and ETEC were the most common bacterial pathogens isolated from 29%, 21% and 7%, respectively. None of Shigella was detected. C.jejuni 36 was the most frequently isolated serotype. Campylobacter isolates were 87% resistant to both nalidixic acid and ciprofloxacin and 6% resistant to azithromycin. No resistance was observed among Salmonella and ETEC strains against ciprofloxacin.

Immunology data is being analyzed.

e. Future plans:

Similar study with more in details immunological assays will be done in year 2004.

10. Title of the project: "Safety, dose, immunogenicity, and community transmission risk of a candidate s.flexneri 2a vaccine among young children in rural Bangladesh".

a. Investigators:

Daniel W. Isenbarger (WRAIR); Abdullah H. Baqui (ICDDRB, Bangladesh), Dr. David N. Taylor (WRAIR), Dr. Thomas L. Hale (WRAIR), Dr. Malabi Venkatesan (WRAIR), Orntipa Sethabutr ,Carl J. Mason and Professor Robert E Black, Dept. of International Health, Johns Hopkins University.

b. Objectives:

To evaluate SC602 safety, fecal shedding, dose and immunogenicity in up to 78 children aged 12-35 months initially in inpatient followed by in outpatient trials at the Matlab field station in Bangladesh.

c. Methods:

Enzyme linked immunosorbent assay (ELISA) method was used to determine the antigen specific serum IgA and IgG titer in 600 serum samples.

d. Results:

The administration of SC602, an orally administered, live attenuated strain of *Shigella flexneri* 2a, was well tolerated by Bangladeshi childern aged 12 to 35 months of age during the inpatient studies. Fewer than expected children demonstrated shedding of the vaccine strain. Specific IgA and IgG production to the lipopolysaccharide of the vaccine strain as measured by ELISA were also less than expected. The reasons for these findings are not entirely clear.

The measurement of specific IgA and IgG titers in all serum samples (600 samples) were done again at this Department to confirm the immunogenicity-findings.

e. Future plans:

This protocol has been terminated. No further activities are planned for the coming year.

D. Department of Veterinary Medicine AFRIMS FY02 Research Accomplishments

1. Title of research project: Efficacy and limited pharmacokinetic profiles of intravenous artemisinin candidates (artelinic acid (AL), artesunate (AS) or AL-like compounds) using a Plasmodium coatneyi - rhesus monkey model of severe malaria.

a. Department:

Veterinary Medicine (collaboration w/Dept. of Immunology & Medicine)

b. Investigators:

Dr. Montip Gettayacamin, LTC R. Scott Miller, LTC Terrell Blanchard, MAJ Kevin Armstrong, Pranee Hansukjariya, Dr. Paktiya Teja-Isavadharm, MAJ Suping Jiang

c. Objectives:

1) Optimize the *P. coatneyi* model of severe malaria for treatment of severe and complicated malaria 2) Determine the degree and rapidity of parasite clearance of candidate intravenous artemisinins 3) Determine the efficacy of various doses of candidate intravenous arteminisinins for severe *P. coatneyi* malaria in rhesus monkeys.

d. Methods:

A: Animal model: Spleen-intact rhesus monkeys serve as donor animals. They are intravenously inoculated with P. coatneyi stabilate, monitored with serial blood smears to desired parasitemia levels, then blood is collected and aliquotted for IV injection into experimental animals.

B. Experimental animals: Splenectomized rhesus are used since splenectomy is necessary to develop high parasitemia and sequestration leading to severe malaria. After injection of infected blood from a donor, serial blood smears are analyzed until parasitemia reaches >25% and/or one of several other "treatment criteria" are reached. Then the animals are treated with antimalarial compound and subsequent serial bloodsmears are used to determine the rate and completeness of parasite clearance. When/if the infection recrudesces, the animals receive definitive antimalarial treatment with chloroquine HCl.

C. Dose ranging: Multiple dose groups are used initially, to determine the minimum effective dose (MED) of the drug compound. The MED determined is then confirmed in another, larger group of animals. Finally, limited pharmacokinetics and pharmacodynamics of the drug at the MED are determined by HPLC and bioassay procedures, both in infected and non-infected animals.

e. Results (accomplishments during the period of January 2002 - December 2002):

Intravenous AS, the succinamide ester of dihydroartemisinin (DHA), and AL, a hydroxymethyl benzoate ester of DHA, were compared in three-day IV treatment regimens in the *P. coatneyi* - rhesus malaria model. Given the rapid acting nature of these drugs, the primary endpoint was clearance of parasites to below the level of detection with microscopy, with time to 99% clearance (PCT99), time to recrudescence, clinical response and cure as secondary endpoints. 100% clearance was achieved with an AS dose of 8 mg/kg loading followed by 4 mg/kg daily for two days. Clearance was confirmed in 10 animals in the uncomplicated model with recrudescence in all animals after a mean of 6 days. 100% clearance was not achieved with AL; the most effective and well-tolerated dose was 11.8 mg/kg loading dose. When this dose was given to monkeys with uncomplicated malaria, only 25% cleared parasitemia.

f. Future plans:

Since the validity and usefulness of this animal model of severe malaria has been well-established, future use of this model in testing of intravenous antimalarial drug candidates may be performed.

2. Title of research project: Toxicology of Single and Subacute Dosing of Candidate Intravenous Artemisinins in Rhesus Monkeys

a. Department:

Veterinary Medicine (collaboration w/Dept. of Immunology & Medicine)

b. Investigators:

LTC Terrell Blanchard, LTC R. Scott Miller, Dr. Montip Gettayacamin, MAJ Kevin Armstrong, Pranee Hansukjariya, Dr. Paktiya Teja-Isavadharm, Dr. Sathit Pichyangkul, MAJ Suping Jiang, Dr. James Petras, MAJ Steven Mog

c. Objectives:

1) Through dose ranging, establish the no effect dose, and minor and major organ toxicities of these drugs given daily for 7 days. 2) Through limited dose ranging, establish the acute, single maximum tolerated dose (MTD) of both intravenous artelinic acid and intravenous artesunate. 3) Determine the cerebrospinal fluid (CSF) levels of the drugs/metabolites which are associated with lethal toxicity in rhesus, and compare these to concurrent blood levels and neurohistopathology.

e. Methods:

Two sequential toxicity studies were performed with both artesunate (AS) and artelinate (AL):

1) Acute single maximum tolerated dose: For each compound, initially 2 monkeys were given IV injections of drug, at three day intervals, with each successive dose being twice that of the previous dose. This was continued until at least one criterion of severe toxicity was reached, and an MTD was estimated. Subsequently, this MTD was confirmed in a cohort of 2 monkeys, to confirm that the initial toxicity was not due to cumulative dosing effects.

2) Subacute 7-day toxicity study: A dose-ranging schedule was used; beginning with 1/2 the MTD calculated from Phase I, doses were given IV for 7 days, then animals were observed 14 days before euthanasia and perfusion fixation. Complete necropsies were performed on all animals. Subsequent doses used were equivalent to half the previous dose; this was continued until a minimal effect dose and a no effect dose could be reached. Serum biochemical analyses, hematologic parameter analysis, and clinical observations were taken at prescribed intervals. CSF was collected on day 7 and day 22.

f. Results (accomplishments during the period of January 2002 - December 2002):

Both compounds exhibited a variety of organ toxicities at high doses, including neurologic, gastrointestinal, renal, and hematologic effects. The apparent pattern of toxicity is different, e.g. AS had more gastrointestinal effects, whereas AL had more renal effects (among others). Overall, however, taking into account the entire study, both compounds were found to be roughly equivalent in their toxicity potential. These results were used along with all other preclinical study data in animals to assist in the down-selection process between the two compounds. Ultimately artesunate was chosen to be the candidate drug for additional efforts leading to an IND.

g. Future plans:

Detailed histopathologic analysis of the brains of the animals used in Phase II is currently on-going at WRAIR; full evaluation of those tissues will likely involve a pathologist at AFRIMS as well.

3. Title of research project: Care and Maintenance of Rhesus (Macaca mulatta) and Cynomolgus (Macaca fascicularis) monkeys and Management of Breeding Colonies.

a. Department:

Veterinary Medicine

b. Investigators:

Dr. Montip Gettayacamin, Mr. Srawuth Komcharoen

c. Objectives:

Maximize the production of specific pathogen-free rhesus and cynomolgus monkeys in the USAMC-AFRIMS production colony, using the best and most humane husbandry care, maintenance procedures, veterinary care, and disease surveillance and environmental enrichment procedures available.

d. Methods:

USAMC-AFRIMS maintains a breeding colony of rhesus and cynomolgus macaques using a closed colony system. Approximately 250 rhesus and 50 cynomolgus monkeys are used in the breeding program. Two types of breeding is managed: compatible male and female pairs are housed in special paired-type caging, and multiple harem groups are established and maintained in large gang cages. Harems consist of one breeding sire and 5-15 adult females. Newborn monkeys are weaned at approximately 6 months of age, and then are reared to adulthood in gang cages with other weanlings. All colony primates are tested routinely for the presence of infectious diseases that pose a threat to either the health of the colony or to personnel working with the primates. Humane use of the animals is assured by the intense oversight of the Institutioanl Animal Care and Use Committee. Veterinary and technical care is extensive and continuous.

Whenever possible, animals are re-utilized in multiple protocols in order to optimize the use of this limited and essential resource.

e. Results (accomplishments during the period of January 2002 - December 2002):

Approximately 60 rhesus and 10 cynomolgus offspring were produced in 2002.

f. Future plans:

Maintain and expand the colony by obtaining 10-15 new breeding males, increasing the number of paired housing cages, and placing breeding pairs in these new cages into additional animal rooms in the vivarium.

4. Title of research project: Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance / Quality Surveillance Program.

a. Department:

Veterinary Medicine

b. Investigators:

Dr. Montip Gettayacamin, Ms. Anchalee Tungtaeng

c. Objectives:

Maintain a breeding colony of specific pathogen-free laboratory rodents to meet the scientific research needs of the USAMC-AFRIMS, using state-of-the-art knowledge, equipment, and facilities.

d. Methods:

USAMC-AFRIMS maintains breeding colonies of laboratory rodents to meet the needs of AFRIMS research. Using state-of-the-art equipment, knowledge, and facilities, production is matched to the anticipated needs of individual research projects. Extensive and thorough recordkeeping ensures that outbred strains remain outbred, and that inbred strains remain truly inbred. An extensive quality assurance/quality surveillance program, which includes serologic assessments as well as necropsy/histopathologic analysis, ensures that the colony produces only high-quality disease-free animals. When necessary, new breeder stock is procured from a reliable vendor in the United States or Japan. Veterinary and technical care is extensive and continuous.

e. Results (accomplishments during the period of January 2002 - December 2002):

Approximately 2200 litters of ICR mice (*Mus musculus*) were produced, comprising a total of over 30,000 mouse pups. Approximately 160 litters of hamsters (*Mesocricetus auratus*) were produced, comprising a total of over 1500 offspring.

Quality assurance evaluations were carried out using 20 ICR mice and 18 hamsters.

f. Future plans:

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free rodents to support USAMC-AFRIMS research needs.

5. Title of research project: A Plasmodium berghei-Mouse Model for Screening Antimalarial Drugs.

a. Department:

Veterinary Medicine

b. Investigators:

Dr. Montip Gettayacamin, Pranee Hansukjariya, Anchalee Tungtaeng

c. Objectives:

To evaluate potential antimalarial chemotherapeutic agents in the *P. berghei* ICR mouse - the modified Thompson Test model.

d. Methods:

The test system used for the determination of antimalarial activity of the compounds is a modification of the suppressive test known as the Thompson Test. Typically in this test, up to 22 groups of 8 mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided from donor mice. On experiment day 0, the donor mice are anesthetized then exsanguinated via cardiac puncture, the blood pooled and the level of parasitemia determined. The pooled blood is then diluted with normal mouse serum to a concentration of 1×10^6 P. *berghei*-infected erythrocytes per inoculum (0.1 ml). The groups of experimental and control mice are inoculated with this parasitized blood on day 0. On day 3, 4, and 5 mice are treated with either the candidate antimalarial drug or with vehicle alone, to serve as the negative control. The drug is administered orally (PO), subcutaneously (SC), intramuscularly (IM), and/or intraperitoneally (IP) up to three times a day, based on the individual and unique pharmacodynamics of the test compound. Each experimental group receives a different dose level, with up to 7 different dose groups per compound. A standard antimalarial drug may be tested along with the candidate drug for structure-activity determination and for quality assurance of the model. Blood films and body weights are taken on the third and sixth days post-infection, then at weekly intervals though day 60. Blood films are stained, examined by light microscopy, and the percent parasitemia determined. All mice are observed twice a day to assess their clinical signs. All mice with negative smears at 60 days are considered cured.

d. Results (accomplishments during the period of January 2002 - December 2002):

Twenty experiments were performed, in which a total of 21 compounds were tested for their potential antimalarial efficacy.

e. Future plans:

This mouse model for screening new candidate antimalarial compounds has been used for over 30 years and is very effective for making comparisons between drugs. It is rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in higher mammalian hosts, including humans. This is a core capability of the USAMC-AFRIMS Department of Veterinary Medicine and will be maintained so that many more compounds can be tested.

E. Department of Virology, AFRIMSFY02 Research Accomplishments

1. Title of research project: The Dengue Hemorrhagic Fever Project II: Continued Prospective Observational Studies of Children with Suspected Dengue

a. Investigators:

1. Principal Investigators:

1. Siripen Kalayanarooj, MD (Queen Sirikit Institute of Child Health, Bangkok)
2. Mammen P Mammen Jr, MD, MAJ (P), MC (USAMC-AFRIMS)

2. Associate Investigators:

1. Ananda Nisalak, MD
   (USAMC-AFRIMS)
2. Khin Saw Aye Myint, MD
   (USAMC-AFRIMS)
3. Supamit Chunsuttiwat, MD
   (Division of General Communicable Diseases, CDC, MOPH)
4. Suntaree Ratanach-eke, MD
   (Queen Sirikit Institute of Child Health, Bangkok)
5. Paisal Lerdluedeeporn, MD
   (Queen Sirikit Institute of Child Health, Bangkok)
6. Vanya Chansiriwongs, MD
   (Queen Sirikit Institute of Child Health, Bangkok)
7. David W. Vaughn MD, MPH, LTC, MC
   (WRAIR)
8. Daniel Libraty, MD
   (Division of Infectious Disease & Immunology, UMASS)
9. Sharone Green, MD
   (Division of Infectious Disease & Immunology, UMASS)
10. Alan L. Rothman, MD
    (Division of Infectious Disease & Immunology, UMASS)
11. Francis Ennis, MD
    (Division of Infectious Disease & Immunology, UMASS)
12. Vichitra Hemsrichart, MD
    (Institute of Pathology, Dept. of Medical Services, MOPH)
13. Henry A.F. Stephens, PhD
    (Siriraj Hospital, University College London Medical School)
14. Rebecca Rico-Hesse, PhD
    (Southwest Foundation for Biomedical Research, Texas)

b. Objectives:

This study continued to define the pathophysiology of illness resulting from dengue infection. Information gained from this study provided important insight into the methods of preventing and intervening in severe dengue disease. The project encompassed studies from 1999 to 2002.

c. Study Specific Objectives:

1. Determine if serologic markers of dengue infection correlate with disease severity and outcome
2. Determine whether the proportion of circulating mononuclear cells infected with dengue virus correlate with clinical outcome
3. Determine if bioimpedance measurements can predict early plasma leakage and the development of shock
4. Determine if apoptosis is an important immune regulatory function capable of down regulating the immune response leading to the development of DHF
5. Develop an in situ PCR for dengue.
6. Determination of neutralizing antibody and memory T-cell responses
7. Characterizing the histopathologic changes and distribution of dengue antigen in various tissues of fatal DHF cases
8. Determine the dengue virus nucleic acid sequences from viruses isolated from patients with mild and severe disease
9. Seek an association between class I or II HLA haplotypes and the severity of secondary dengue infections and disease
10. Determine clinical predictors of DHF to include age, sex, weight, nutritional status, tourniquet test, clinical symptomatology, and liver enzyme abnormalities. These clinical parameters will be examined by DHF grade and infecting dengue serotype.

d. Methods:

Children were enrolled if they were suspected of having an early DV infection (without evidence of DHF) or a fever without an identifiable source. Inclusion criteria included an oral temperature $\geq 38.5°C$, fever onset not longer than 72 hours prior to the initial evaluation, weight > 6kg, flushed face, signed consent by parent or guardian. After informed consent was obtained, subjects were admitted to the hospital and a blood specimen obtained. The result of the plasma test for DV RNA by RT-PCR was available the morning of study day 2. Children who were DV RT-PCR-negative were given the opportunity to leave the study, or to continue in the study for clinical observation. Those children remaining in the hospital underwent inpatient observation until one day following defervescence (fever day +1). Clinical information was collected and recorded daily. Serial blood samples were collected and analyzed for

e. Routine and dengue-specific blood and plasma tests were conducted to include:

1. CBC, WBC differential, AST, Albumin

2. Hemagglutination inhibition (HAI) assay for dengue
3. Antibody-capture DV IgM/IgG enzyme immunoassay (EIA)
4. RT-PCR for dengue, Plasma viremia titers
5. Dengue virus isolation in Toxorhynchites splendens and typing
6. IL-15, IL-18, MIP-1a, MIP-1b, and MCP-1, CD69, CD38, and Ki-67
7. Labeled antibodies to identify T cell subsets, NK cells and B cells
8. NS1 (soluble NS1 and anti-NS1 antibodies)
9. Complement assays

f. Results (January 2002 - December 2002):

Seventy-six (76) children were enrolled, 24 (31%) dengue cases were diagnosed based on EIA/HAI serology, and 2 (8%) experienced a primary infection. PCR identified 100% (24/24) of the cases. The breakdown by serotype included 12 DEN-1, 10 DEN-2, and 2 DEN-3.

g. Future plans:

1. Completion of specimen processing and testing.
2. Perform data analysis.
3. IRB approval and initiation of a new DHF study continuing our enrollment of children experiencing dengue infections, stratifying the infections by severity, correlating serologic markers of dengue infection with severity of disease, and initiating novel methods of measuring and quantitating plasma leakage.

2. Title of research project: A Recombinant Hepatitis E Vaccine Efficacy Study In Nepalese Volunteers

a. Investigators:

1. Principal Investigators:

1. M. P. Shrestha (WARUN)
2. R. M. Scott (WARUN)

2. Associate Investigators:

1. G. B. Thapa (SBH)
2. K. S. A. Myint (AFRIMS)
3. R. A. Kuschner (WRAIR)
4. D. M. Joshi (SBH)
5. M. P. Mammen (AFRIMS)
6. B. L. Innis (GSK)

b. Objectives:

To evaluate the protective efficacy for the prevention of hepatitis E disease provided by the candidate hepatitis E vaccine administered according to a 0, 1 month schedule with a booster dose at month 6.

c. Study Specific Objectives:

(see below)

d. Methods:

(see below)

e. Results (January 2002 - December 2002):

(see below)

A candidate recombinant baculovirus expressed hepatitis E virus (HEV) vaccine was found to be safe and immunogenic in 88 American and 44 Nepali volunteers. A 20 μg formulation was selected for further evaluation in a randomized double blind placebo controlled efficacy trial in susceptible, active duty Royal Nepal Army volunteers. Of 5,263 consenting volunteers screened, 3,113 were susceptible to HEV. Two thousand volunteers (5 females, 1,995 males) were enrolled, receiving either placebo or 20μg of active vaccine. Volunteers were vaccinated at 0, 1, and 6 months with sera collected at months 0, 1, 3, 6, 7, 13, and 24. Sera were examined for HEV total immunoglobulin (HEV Ig) by an enzyme-immune-assay, to determine the immunogenicity of the vaccine over time. One tenth of the volunteers were followed on days 1, 3, 5, and 7 after each vaccination for local and general solicited adverse events (SoAE). Non-serious adverse events (NSAE) were recorded for 30 days after each vaccination and serious adverse events (SAE) were to be collected throughout the 2-year study period. Sera and stool from cases meeting clinical and biochemical criteria compatible with viral hepatitis, were examined for HEV RNA by a reverse transcriptase-polymerase chain reaction, and serologically for HEV Ig, HAV IgM, HbsAg, HbcIgM and HCV IgG.

SoAE averaged <50% in 194 volunteers following all vaccinations. The most common SoAE was mild pain at the injection site. With over 550 NSAE, 2 with injection-site pain were related to vaccination. To date, 250 SAEs resulted from unrelated hospitalizations and deaths. Five hospitalizations, one day after the first vaccination, might have been related to vaccination.

One thousand seven hundred and ninety four (1,794) volunteers received a three vaccine series. Forty-four (44) HEV cases occurred during the following rainy season. Unblinding these cases will occur in early 2003 and allow for determinations of vaccine efficacy.

f. Future plans:

Additional clinical studies planned on safety and immunogenecity in adult females, adolescents and in chronic liver disease patients.

3. Title of research project: Prospective Study of Dengue Virus Transmission and Disease in Primary School Children

a. Investigators:

1. Principal Investigators:

1. Supamit Chunsuttiwat MD, MPH (Ministry of Public Health [MOPH])
2. Mammen P. Mammen Jr, MAJ, MD, MC (USAMC-AFRIMS)

2. Associate Investigators:

1. Ananda Nisalak, MD
   (USAMC-AFRIMS)
2. David W. Vaughn, MD, MPH, COL, MC
   (WRAIR)
3. Virat Bhudimethi, MD, Chief, Medical Office, Kamphaengphet
   (MOPH)
4. Daniel Libraty, MD
   (Division of Infectious Disease & Immunology, UMASS)
5. Alan L. Rothman, MD
   (Division of Infectious Disease & Immunology, UMASS)
6. Sharone Green, MD
   (Division of Infectious Disease & Immunology, UMASS)
7. Francis Ennis, MD
   (Division of Infectious Disease & Immunology, UMASS)
8. Henry A. F. Stephens, PhD
   (Division of Nephrology, Institute of Urology and Nephrology, University College London Medical School)

b. Objectives:

To improve our understanding of the immunopathology of dengue virus infections by prospectively comparing immunological responses of asymptomatic and symptomatic dengue infections. The immunological responses measured include humoral (neutralizing [protective] and antibody-dependent enhancement) antibody and memory T and B cell responses. In addition, we sought to elucidate additional factors, which may contribute to the development of symptomatic and asymptomatic dengue infections, such as HLA type, nutritional indices, and immunoglobulin subclass responses.

c. Study Specific Objectives:

1) Developing a prospective cohort population of 2, 000 school-aged volunteers at risk for dengue infection.

2) Developing a bank of plasma and PBMC samples from this population prior to the start of the dengue season.

3) Identify subgroups of individuals who are dengue infection naïve and immune

4) Follow the cohort prospectively for five years with scheduled sampling of plasma and T-cells, monitor for the occurrence of febrile illnesses within the cohort to capture acute dengue infections, and perform serum sampling of those experiencing an acute dengue infection.

5) To identify individuals experiencing asymptomatic dengue infections.

d. Methods:

This was a prospective school based study of about 2,000 children, which began in January 1998 and ended in December 2002. Students from 12 schools, Amphur Muang, Kamphaengphet province in grades 1 to 5 were enrolled into the study in January1998. Baseline demographics, anthropometric measurements, and plasma and T-cells were obtained. A similar baseline evaluation, including anthropometric measurements, occurred every January for the duration of the study. Incoming first grade classes were enrolled and students were followed until the completion of the sixth grade. Scheduled serological evaluations of the entire cohort were performed on June 1st, August 15th and November 1st of each study year. Active case surveillance of participating schools, using the criteria of missing greater than or equal to 1 day of school or reporting illness to the school health nurse, commenced at the beginning of the dengue season on 1 June and continued until the end of the dengue season, 1 November, for each year. Village health workers tracked school absences and school illnesses, made first contact and completed a survey of symptoms, and obtained and documented an oral temperature. Children with a fever or history of fever within seven days of any school absence provided a venous blood sample at the time of their illness (public health nurse) and a convalescent sample 14 days later (by a study nurse).

e. Results (January 2002 - December 2002):

In January 2002, 1,372 students were enrolled in the study. The dropout rate for the start of the 2002 surveillance was 8%. The mean drop out rate over 5 years was 9%, primarily due to the movement of families out of the surveillance schools. The gradual decline in the study population at the start of active surveillance declined from 2,119 in 1998 to 1,372 in 2002 and is a reflection of the changing demographics of the surveillance schools, a decrease in the size of second grade classes, and a transient decline in the communities interest in the study.

There were 27 acute secondary dengue infections during the surveillance period. Three cases were hospitalized representing DHF grades I, II, and III.

Dengue virus was detected by PCR in 92.5% of serologically confirmed cases. DEN-2 was the predominant serotype (60%); DEN-1 and DEN-4 were detected at the same rate (20% each). There were no DEN-3 cases detected.

f. Future plans:

1. Continued processing of samples collected in 2001 and 2002.
2. Perform a data Analysis.
3. Completion of a new, 2-story research facility, which will be utilized for clinical and laboratory operations related to the prospective, and related, studies.
4. IRB approval and initiation of a new prospective study continuing dengue infection surveillance in the KPP region, further defining the epidemiological trends of dengue infections in Thailand, and expansion of the recently completed study to include village cluster and vector investigation and surveillance.

4. Training and Workshops

a. Background:

The Department of Virology, Armed Forces Research Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand, seeks to expand its diagnostic capabilities in Southeast Asia by improving regional laboratory capabilities through the dissemination of diagnostic kits and the training of technical personnel.

b. Goals:

1. To create and improve the laboratory infrastructure of Southeast Asian regional laboratories specializing in infectious disease surveillance.
2. To provide the training of laboratory personnel (technicians and supervisors) working in Southeast Asia and beyond in infectious disease diagnostic techniques.

c. Activities:

1.The Defense Threat Reduction Agency (DTRA) requested a 3-week training block at the Department of Virology, USAMC-AFRIMS, for the Director and 9 physician scientists from the Institute of Virology, Tashkent, Uzbekistan. Individual training was provided, utilizing translators, in the techniques of ELISA, PCR, HAI, neutralization assay, tissue culture, and sequencing.

2.Dr. Bui Nghia Vuong, a researcher at the National Institute of Veterinary Research, Hanoi, Vietnam, received a 3-week training program in Japanese encephalitis (JE) diagnostic testing to include ELISA, HAI, neutralization assay, and tissue culture.

3.Over 50 student scientists from Srinakarinviroj University, Chulalongkorn University, Phramongkutklao Medical College and Faculty of Tropical Medicine, Mahidol

University received 2 to 4 weeks of training in the diagnostic modalities employed at the Department of Virology, AFRIMS.

5. Febrile Disease Surveillance, Kathmandu, Nepal

a. Background:

The Dept. of Virology, AFRIMS, and the Walter Reed Army Research Unit, Nepal (WARUN) field office, have completed several cohort studies on hepatitis E virus (HEV) over the past 16 years. These efforts have established excellent rapport with the health care providers at the Teku and Shree Birendra Hospitals. AFRIMS continues collaborations with the Environmental Health Project (EHP) and Nepal Ministry of Health (MOH) on the study of flavivirus seroprevalence in Terai, the area of tropical lowlands across the southern portion of Nepal bordering India. Terai is a breeding area for vectors, which transmit malaria, kala-azar, and JE. Fever surveillance in Nepal this past year focused on encephalitic, hemorrhagic, and icteric syndromes. While the incidence of the infectious diseases responsible for these syndromes appears to be increasing, there is no organized national surveillance program in Nepal to monitor for febrile illnesses or emerging infectious diseases.

b. Goals:

1. To determine if the etiologies of fever in travelers and populations indigenous to Nepal are emerging diseases e.g., viral hepatitis, dengue, JE, leptospirosis, and scrub typhus.
2. To establish a surveillance system in selected hospitals and points of health care delivery for the purpose of defining the epidemiologic and clinical characteristics of encephalitis in Nepal.
3. To determine the incidence and etiology of vector-born febrile illnesses in Nepal.

c. Methods:

A prospective surveillance system was established for monitoring icteric, encephalitic, and hemorrhagic illnesses at selected hospitals and institutions in Kathmandu and in the Terai. Patients 18 years or older with acute febrile illnesses meeting the following criteria were enrolled; 1) an illness with 2 or more hemorrhagic manifestations such as epistaxis, bloody stool, purpural rash, hemoptysis, hematemesis, hemorrhage into mucous membranes, or hematuria; 2) those with a clinical diagnosis of encephalitis or a presentation of headache, mental status changes, seizures, and/or neck stiffness without an identifiable bacterial cause; and 3) those with a clinical diagnosis of hepatitis or an alanine aminotransferase (ALT) 2.5 times the upper limit of normal or a peak total bilirubin level greater than 2 mg/dl. When possible, both acute and convalescent sera were drawn from each patient 10-14 days apart. Those with fever for more than 7 days were excluded from the study. Demographic and clinical data were gathered at the surveillance hospitals. Sera and CSF, in the cases of encephalitis, were tested at AFRIMS by

enzyme linked immunosorbent assay (EIA) for antibodies to JE, dengue, Leptospira, and scrub typhus. Those presenting with icterus were screened for Hepatitis A, B, C, and E by an EIA panel and by polymerase chain reaction (PCR) for HEV. All data were compiled, documented, and analyzed at AFRIMS.

d. Progress:

A total of 1,063 specimens (sera and CSF) from 963 patients presenting with encephalitis were collected at the Vector-Borne Disease Research and Training Center (VBDRTC), Bheri Zonal Hospital (BZH) and B.P. Koirala Institute of Health Sciences (BPKIHS) and tested using the anti-JE IgM/IgG ELISA at the Department of Virology, AFRIMS.

EIA and HEV PCR were completed on 130 cases of febrile jaundice; approximately one-half (46%) were confirmed to have Hepatitis E infection. The prevalence of viral hepatitis other than E was very low in this community.

There were 3 confirmed dengue infections (two primary and one secondary dengue) in Kathmandu. Dengue is not endemic in Nepal, the details of travel to dengue-endemic regions is unknown.

6. Hospital-based EID Surveillance, Kamphaeng Phet, Thailand

a. Background:

Kamphaeng Phet Provincial Hospital (KPPH) is located in northwestern Thailand and serves urban and rural communities with a large population of hill-tribe Thai and Burmese. This environment offers unique opportunities to study and identify emerging infectious diseases with the potential to significantly impact regional and national health issues.

b. Goals:

1. To characterize the etiologies of fever and hepatitis, encephalitis, and hemorrhage in defined geographical regions of northwest Thailand.
2. To investigate the public health impact of the etiologies of fever and hepatitis, encephalitis, and hemorrhage.
3. To expand and improve the scientific and logistical infrastructure of KPP by completing construction, and initiating operations, in a new 2-story building outfitted with clinical and laboratory resources.

c. Methods:

This is a hospital-based study closely related to the study of febrile diseases in Nepal. Focus will be on presentations of febrile illness with icteric, hemorrhagic, or encephalitic manifestations. All the case definitions, surveillance, and laboratory methods, specimen

collection, and data processing are similar. Inclusion criteria are also similar, except for the absence of age limitations. Illnesses with an onset more than 7 days previous were excluded. Sera were screened at the field hospital using a rapid dipstick for leptospirosis, scrub typhus, and murine typhus. All those positive by dipstick were confirmed using an IgM ELISA completed at AFRIMS.

d. Progress:

Approximately 1,200 cases presented with one of the specified clinical syndromes. One-third (33 out of 100) of febrile cases screened positive by IgM EIA and 61% (638 out of 1,048) were serologically confirmed to be dengue. Hepatitis B virus was the leading cause of admission for a hepatitis syndrome (18% or 8 out of 43).

7. Influenza Surveillance in Southeast Asia

a. Background:

Influenza is an important cause of morbidity and mortality among populations at the extremes of age. Continuous viral surveillance and isolation of influenza viruses provides important information for the creation of annual vaccine formulations based on the identification of new and emerging strains of influenza.

b. Goals:

1. To provide isolates of influenza virus collected in Southeast Asian countries as part of the global surveillance network for influenza, " Project Gargle".
2. To evaluate rapid diagnostic techniques at select sentinel sites in an attempt to validate these tests, increase sample submissions, and improve patient care.
3. To expand the network of participating institutions to include the U.S. Embassies of Southeast Asia.

c. Methods:

Samples were collected from patients with clinically suspected influenza infection based on the fulfillment of the case definition (fever or history of fever $\geq$38°C and two or more of the following symptoms: cough, sore throat, coryza, muscle aches, malaise/fatigue, or headache). Clinical history forms, inclusive of basic demographic and clinical information, were completed by the OPD nurse or AFRIMS research nurse. Throat swabs were collected, placed in viral media, and stored at -70°C. All specimens were shipped on dry ice to AFRIMS, which in turn shipped the samples to Armstrong Laboratory, Brooks AFB. Rapid diagnostics for Influenza (FLU OIA) were field tested at select sites. Laboratory test results were maintained and summarized by "Project Gargle" and CDC personnel.

d. Progress:

AFRIMS submitted 150 samples to Brooks AFB from October 2001 through October 2002; 8 from the US Embassy, Bangkok; 51 from KRCH Sangkhlaburi; 11 from CIWEC Kathmandu; 77 from Kamphaeng Phet; and 3 from the Maldives. Viral isolation and serotyping of the 10 specimens from the Maldives were completed by the Faculty of Medicine, Siriraj Hospital.

III. APPENDICES:

A. PERSONNEL ASSIGNED UNDER AGREEMENT

Department of Administration

1. Ms. Bang-on Kesdee
2. Mr. Weerasak Yeephu
3. Mr. Sompol Boonnak
4. Ms.Pattrapan Jullasing
5. Mr. Puwanai Sangsri
6. Ms.Geerati Sornwattana
7. Mrs.Wichayada Wattanatom

Department of Logistics

8. Mrs.Sutthida Srijan
9. Mr.Nipat Promchart
10. Mr.Somchai Putasang
11. Mr.Mongkol Puramast
12. Mr.Surapol Ogpai
13. Mr.Sawadi Boonnak
14. Mr.Charan Kajeechitr
15. Mr.Thongchai Duangkeaw
16. Mr.Boonthum Jamjank
17. Mr.Komson Boonnak
18. Mr.Somporn Pinpo
19. Mr.Chatchai Sang-ngen
20. Mr.Nirutti Boonnak
21. Mr.Panutat Inthamattayakul
22. Mr.Prasitchai Kruysawat
23. Mr.Yuthana Seemat
24. Mr.Sawet Amnuay
25. Mr.Sanchai Wanichsan
26. Ms.Prawitchaya Kumhoon
27. Ms.Nawaporn Chantakulkij
28. Ms. Yaowalux Kitkungwal

Department of Immunology

29. Mrs. Barnyen Permpanich
30. Ms. Utaiwon Kum-arb
31. Mrs.Mali Ittiverakul
32. Ms. Amporn Chalouydumrong
33. Ms. Nillawan Buathong
34. Mr. Chaiyawat Mathavarat
35. Mr. Prasit Sookto
36. Ms.Nitima Chanarat
37. Mrs.Somchit Tulyayon

38. Ms.Noojcharin Labhantakul
39. Ms.Srisombat Wannaying
40. Ms.Apassorn Saelim

Department of Virology

41. K.Y Ananda Nisalak
42. Ms.Russama Jittawisutthikul
43. Mrs. Chuanpis Ajariyakhajorn
44. Mrs. Sumitda Narupiti
45. Mrs. Naowayubol Nutkumhang
46. Mrs. Vipa Thirawuth
47. Ms. Panor Srisongkram
48. Ms. Nawarat Charoensri
49. Ms. Patama Monkongdee
50. Mr. Surind Sisiranond
51. Mr. Somsak Imlarp
52. Mr. Pairote Tararut
53. Mr. Pongpun Sawatwong
54. Mr. Wichien Sa-Nguansuk
55. Mr. Prachakkra Panthusiri
56. Mr.Thongchai Khainkaew
57. Ms.Warinda Srikam
58. Ms.Wallika Kulthongkum
59. Mr.Wamchai Inpho
60. Mrs.Pannarat Chuakanubon
61. Mrs.Rungkarn hangsuwan
62. Dr. Robert McNair Scott
63. Dr.Mrigendra Prasad Shrestra
64. Dr.Henry A.F. Stephens

Department of Veterinary Medicine

65. Mr. Komdej Kongsunarat
66. Mr.Phongsak Maneerut
67. Mr.Suchin Poolgird
68. Mr.Thonglor Detkokao
69. Mr.Sawaeng Sripakdee
70. Mr.Phatcharaphon Jaikla
71. Mr.Samruay Jecksaeng
72. Mr.Dejmongkol Onchompoo
73. Mr.Manop Pooyindee
74. Ms.Choosri Sangsri
75. Mr.Charin Kheowcharas
76. Mr.Srawuth Komcharoen
77. Ms.Anchalee Tungtaeng

78. Mr.Surayuth Seegaewin
79. Mr.Somkid Tosawong
80. Mr.Manas Kaewsurind
81. Mr. Yongyuth Kongkaew
82. Mr.Vittavat Sankalee
83. Mr.Mana Saithasao
84. Ms.Anchalee Pothipoch
85. Mr.Bamrung Chaikwang
86. Ms.Siriwan Korpaiboonkij
87. Mr.Alongkorn Hanrujirakomjorn
88. Mr.Sornchai Jansuwan
89. Mrs.Intira Jareonwatanan
90. Mr.Amnaj Andang
91. Mr.Suvit Boonkali
92. Mr.Sarayuth Chienrum
93. Mr.Sakda Sanon
94. Ms.Kwanpracha Insansueb

Department of Entomology

95. Ms. Jula Nathong
96. Mr.Prasan Kankaew
97. Mr. Chukree Kaittibut
98. Mr. Nattapat Nongngork
99. Ms.Nongnuch Yimamnuaychok
100. Ms.Nattawan Ratchapraw
101. Ms. Kalyakorn Wongkalasin
102. Mr. Punnarat Kertmanee
103. Mr. Somsak Tiang-trong
104. Ms.Kanchana Pantuwatana
105. Ms. Bousaraporn Tippayachai
106. Mr.Sommai Promsathaporn
107. Ms.Sasathorn Nongngork
108. Mr.Opas Thachin
109. Ms.Warisa Leepitakrat
110. Mr.Boonsong Jaichapor
111. Ms.Werawan Chonarom
112. Mr.Tanapone Laohachainam
113. Mr. Chalermpon Kumpitak
114. Dr.Rampa Rattanrithikul
115. Mr.Kirkvich Chandranoi
116. Ms.Jaruwan Tawong
117. Ms.Rachaneeporn Jenwithisuk
118. Mr.Somporn Chanaimongkol
119. Mr.Weeraphan Kongtak

120. Mr.Vajira Auevanich
121. Mr.Inkam Inlao
122. MG.Vichai Sangkasuwan
123. Ms.Koraket Laptaveechoke
124. Ms.Sucheera Insuan
125. Mr.Weerayut Chareonsongsermkit
126. Ms.Nittaya Khlaimanee
127. Mr.Narong Ponsa
128. Ms.Nongnuch Maneechai
129. Mr.Udom Kijchalao

Department of Enteric Diseases

130. Mr. Songmuang Piyaphong
131. Ms.Duangjai Lumson
132. Ms.Ovath Thonglee
133. Ms.Sasikorn Silapong
134. Ms.Rungnapha Phasuk
135. Ms.Anuchittada Sirisriro
136. Ms.Chittima Pitarangsi
137. Mr.Boonchai Wongsatitiwilairoong
138. Ms.Ajchara Aksomboon
139. Ms.Nucharee Thongsen
140. Mrs.Wonlana Teerapolumpun
141. Mr.Supin Pankote
142. Mr.Suchart Thepsanan
143. Mr.Prasert Meesuksabai
144. Ms.Prapatcha Chitsoonthornrat
145. Mrs.Tanintorn Adeedto
146. Mr.Papunkorn Puapuek
147. Ms.Rapida Padmasankha
148. Mr.Pakornpat Supanich
149. Mr.Nan Chen
150. Mrs. Tippa Wongstitwiliroong

****

B. Publications 2002

1. Bodhidatta L, Vithayasai N, Eimpokalarp B, Pitarangsi C, Serichantalergs O, Isenbarger DW. **BACTERIAL ENTERIC PATHOGENS IN CHILDREN WITH ACUTE DYSENTERY IN THAILAND: INCREASING IMPORTANCE OF QUINOLONE-RESISTANT CAMPYLOBACTER. Southeast Asian J Trop Med Public Health 2002; 33(4):752-57.**

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